

EDGEWOOD

CHEMICAL BIOLOGICAL CENTER

U.S. ARMY SOLDIER AND BIOLOGICAL CHEMICAL COMMAND

ECBC-TR-229

**IDENTIFICATION, PURIFICATION,
AND PARTIAL CHARACTERIZATION
OF THE GV-DEGRADING ENZYME
FROM ATCC # 29660 *ALTEROMONAS UNDINA***

**Steven P. Harvey
Tu-chen Cheng**

RESEARCH AND TECHNOLOGY DIRECTORATE

February 2002

**Approved for public release;
distribution is unlimited.**



20020429 050

Aberdeen Proving Ground, MD 21010-5424

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

REPORT DOCUMENTATION PAGE			Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.				
1. AGENCY USE ONLY (Leave Blank)		2. REPORT DATE 2002 February		3. REPORT TYPE AND DATES COVERED Final; 00 Oct - 01 Sep
4. TITLE AND SUBTITLE Identification, Purification, and Partial Characterization of the GV-Degrading Enzyme from ATCC # 29660 <i>Alteromonas undina</i>			5. FUNDING NUMBERS P-106013	
6. AUTHOR(S) Harvey, Steven P., and Cheng, Tu-chen				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) DIR, ECBC, ATTN: AMSSB-RRT-BE, APG, MD 21010-5424			8. PERFORMING ORGANIZATION REPORT NUMBER ECBC-TR-229	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)			10. SPONSORING/MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release; distribution is unlimited.			12b. DISTRIBUTION CODE	
<p>The GV (2-dialkylaminoalkyl N,N-dialkylphosphonamidofluoridate) nerve agent has a toxicity intermediate to G and V-type nerve agents and is not catalyzed by either organophosphorus acid anhydrolases (OPAA) or organophosphorus hydrolase (OPH) enzymes. We have screened and identified a number of <i>Alteromonas</i> strains possessing catalytic activity using a GV compound as substrate. The enzyme from one of these strains, <i>A. undina</i>, has been purified to homogeneity by ammonium sulfate fractionation and Q Sepharose anion exchange chromatography. The activity of GV-hydrolyzing enzyme peak is distinct from that of <i>A. undina</i> OPAA following the Q Sepharose column chromatography. The SDS-polyacrylamide gel electrophoresis of the GV-hydrolyzing enzyme fraction revealed a single polypeptide of ~20kDa. To our knowledge, this is the first report of enzymatic detoxification of GV.</p>				
14. SUBJECT TERMS Enzymes Hydrolysis			15. NUMBER OF PAGES 18	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT UNCLASSIFIED		18. SECURITY CLASSIFICATION OF THIS PAGE UNCLASSIFIED		19. SECURITY CLASSIFICATION OF ABSTRACT UNCLASSIFIED
			20. LIMITATION OF ABSTRACT UL	

Blank

PREFACE

The work described in this report was authorized under Project No. 106013, Tech Base Program. This work was started in October 2000 and completed in September 2001.

The use of either trade or manufacturers' names in this report does not constitute an official endorsement of any commercial products. This report may not be cited for purposes of advertisement.

This report has been approved for public release. Registered users should request additional copies from the Defense Technical Information Center; unregistered users should direct such requests to the National Technical Information Center.

Blank

CONTENTS

1.	INTRODUCTION.....	7
2.	MATERIALS AND METHODS	8
2.1.	Growth of Bacterial Strains.....	8
2.2.	Enzyme Assays	8
3.	RESULTS AND DISCUSSION.....	8
3.1.	Spontaneous Hydrolysis - Comparison to G-type Nerve Agents	8
3.2.	Screening of Bacterial Strains and Enzymes for GV Activity	9
3.3.	Purification of GVH.....	10
3.4.	Enzyme Characterization	13
3.4.1	pH Profile.....	13
3.4.2	Temperature Profile.....	14
4.	CONCLUSIONS	15
	LITERATURE CITED	17

FIGURES

1.	Structure of 2-dimethylaminoethyl N, N-dimethylphosphonamidofluoridate.....	7
2.	Reaction Scheme for the Hydrolytic Defluorination of GV.....	9
3.	Purification of GVH on Q Sepharose Column with 0 - 1.0 M NaCl Gradient	11
4.	Purification of GVH on Second Q Sepharose Column	12
5.	SDS-PAGE of Fractions from GV Degrading Enzyme Purification.....	13
6.	pH Profile of GVH.....	14
7.	Temperature Profile of GVH.....	15

TABLES

1.	Spontaneous Hydrolysis Rate of 0.01 M Substrate in 50 mM Bis-tris-propane (pH 7.2) at 25 °C	9
2.	GV Activity (nmol/min/mL) of Lysates	10
3.	Stepwise Elution of GVH Activity.....	11

IDENTIFICATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF THE GV-DEGRADING ENZYME FROM ATCC # 29660 *ALTEROMONAS UNDINA*

1. INTRODUCTION

The enzymes organophosphorus acid anhydrolase (OPAA) from *Alteromonas* sp. JD6.5 and organophosphorus hydrolase (OPH) from *Pseudomonas diminuta* have been shown to catalyze the hydrolysis of a number of toxic organophosphorus (OP) compounds including several G-type chemical nerve agents.¹⁻¹² The OPH also has catalytic activity against V-type nerve agents.¹³ Both these enzymes have been cloned into *Escherichia coli* and can be produced in large quantities. The OPH enzymatic activity for specific substrates has also been increased by genetic manipulation of the cloned gene.¹⁴ The catalytic activity of these enzymes against nerve agents provides considerable potential for decontamination and detoxification of toxic OPs, and/or *in vivo* prophylaxis.

There is another important class of potential threat agents for which catalytic decontamination has not previously been demonstrated. The GV compounds have the generalized structure of 2-dialkylaminoethyl N,N-dialkylphosphonamidofluoridate. An example is 2-dimethylaminoethyl N,N-dimethylphosphonamidofluoridate (Figure 1). The GV compounds are generally much more toxic than G agents and are not included in the Chemical Weapons Convention schedules of compounds.

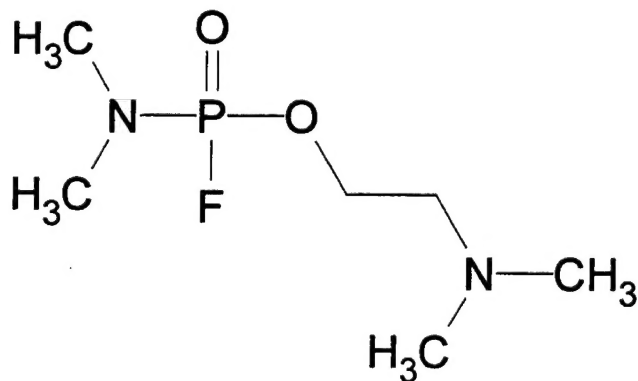


Figure 1. Structure of 2-dimethylaminoethyl
N, N-dimethylphosphonamidofluoridate

Neither OPAA's from *A. sp.* JD6.5 or *A. haloplanktis*, nor OPH have any detectable catalytic activity against compound 1 (unpublished data). To date, there have been no reports on the enzymatic detoxification of GV compounds.

The GV poisoning is also refractory to traditional atropine/ reactivator treatments.¹⁵ Enzymes, however, may offer a successful treatment for GV poisoning. *In vivo* circulating enzymes have already been demonstrated to confer protection against toxic OP agents in mice. The LD₅₀ levels of protection exceed those obtained with atropine/reactivator.¹⁶

Finally, the gene encoding GV enzymes may offer a good basis for protein engineering for improving VX and G-agent activity due to the similarity of GV and VX structures.

2. MATERIALS AND METHODS

2.1. Growth of Bacterial Strains.

Bacteria strains were grown in Instant Ocean Medium consisting of 38 g of Instant Ocean (Aquarium Systems, Mentor, OH), 5 g of proteose peptone, and 1 g of yeast extract per liter at 30 °C for 16-20 hr.

2.2. Enzyme Assays.

Enzyme assays were conducted with a fluoride electrode attached to a Corning 355 pH/ion analyzer. Fluoride release rates were followed until a stable release rate was observed over a period of several minutes. Reactions were conducted in a temperature-controlled vessel in a total volume of 2.5 mL. Background hydrolysis rates (Table 1) were measured to provide a comparison of the relative stability of the different substrates in an aqueous matrix. Background hydrolysis rates were measured by adding neat substrate directly to 2.5 mL buffer solution to a final concentration of 0.01 M. For assaying enzymatic activity, substrates were diluted into isopropanol for the OPAA assays and into methanol for the OPH assays. The reaction medium contained 50 mM Bis-tris-propane (BTP) pH 7.2, 0.1 mM MnCl₂ for OPAA assays and 0.05 mM CoCl₂ for OPH assays, 3 mM GV compound (1), and 1-5 µL of enzyme sample in a total volume of 2.5 mL. For each assay, the substrate was added last. After a 1-4 min equilibration period, a background hydrolysis rate was measured for 4 min. An identical reaction mixture was then monitored in the presence of enzyme and the background values were subtracted. Assays conditions were adjusted to ensure that <10% of the total substrate was consumed in the assay.

3. RESULTS AND DISCUSSION

3.1. Spontaneous Hydrolysis – Comparison to G-type Nerve Agents.

The GV is relatively stable in aqueous solution, as compared to GB and GD. Table 1 illustrates the spontaneous hydrolysis (defluorination) rate of 0.01 M GB, GD and GV at pH 7.2 and 25 °C.

Table 1. Spontaneous Hydrolysis Rate of 0.01 M Substrate in 50 mM Bis-tris-propane (pH 7.2) at 25 °C

Substrate	Rate ($\mu\text{mol/min}$)
GB	58
GD	35
GV	4.0

3.2. Screening of Bacterial Strains and Enzymes for GV Activity.

Because no enzymes have previously been reported to catalyze the hydrolysis of GV, a screening was conducted to search potential sources. The reaction scheme for GV detoxification is illustrated in Figure 2. Fluoride is the leaving group (as HF) and defluorination yields a hydroxylated product (GV-OH). Reactions consisting of 3 mM GF, buffer, and crude cell lysate were monitored by fluoride electrode for the rate of increase in the fluoride ion concentration.

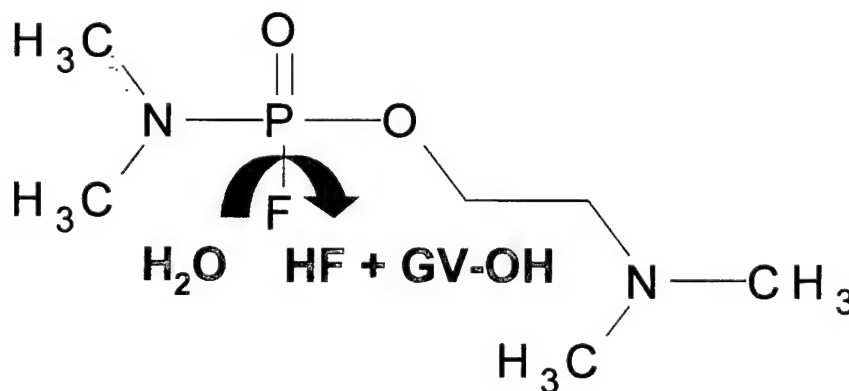


Figure 2. Reaction Scheme for the Hydrolytic Defluorination of GV

Twelve *Alteromonas* strains were used because they have been shown to possess activity against different OP neurotoxins. These *Alteromonas* strains were grown in Instant Ocean Medium, lysed twice in a French press, centrifuged and the lysates obtained were assayed for activity against GV compound (1). Assays were conducted in 50 mM Bis-tris-propane (pH 7.2) at 25 °C. Results are shown in Table 2.

Table 2. GV Activity (nmol/min/mL) of Lysates

ATCC #	Strain	GV Activity	ATCC #	Strain	GV Activity
27127	<i>A. haloplanktis B</i>	169	14393	<i>A. haloplanktis Z</i>	77
23821	<i>A. haloplanktis C</i>	24		<i>A. espejiana</i>	132
19648	<i>A. haloplanktis J</i>	186	33043	<i>A. sp. G</i>	192
	<i>A. haloplanktis M</i>	444	33524	<i>A. sp. M</i>	238
33492	<i>A. luteoviolaceans</i>	5	29332	<i>A. sp. P</i>	40
35358	<i>A. undina</i>	155	19375	<i>A. niger</i>	29

In addition to the lysates shown in Table 2, three different purified enzymes (OPAA cloned from *A. sp.* JD6.5 and *A. haloplanktis*, and OPH from *P. diminuta*) were also tested. Previously, all three of these enzymes had been demonstrated to possess significant activity against several G-type agents.^{7,9,10,11} However, none of the three had any detectable GV activity under the conditions used for the assays (MATERIALS AND METHODS). Limits of detection for the assays were at least in the low nmol/min/mg range.

It should be noted that enzymes with higher specific activity are sometimes obtained from strains with lower crude lysate activity. In addition, the crude lysate enzymatic activity is usually dependent on growth conditions. Based on previous enzyme purification experience with *A. undina* # 35358 strain, it was selected for further purification of its GV-hydrolyzing enzyme(s), designated as GVH.

3.3. Purification of GVH.

A single colony was grown overnight in Instant Ocean Medium (pH 7.0) at room temperature in a 100 mL shake flask. The overnight cultures were transferred to a BioFlow 3000 bioreactor (New Brunswick Scientific Co) with a 6 L working volume of the same medium and cultivated for 7.5 hr at a pH of 7. A total of 53 g wet cells were harvested and resuspended in 200 mL 10 mM bis-tris-propane pH 7.2. After cells were broken in a French pressure cell (SLM Aminco), cellular debris was removed by centrifugation at 5,000 xg. The cell lysate, which contained the GVH activity, was further purified by 40-65% ammonium sulfate fractionation. The pellet was re-suspended in a minimal volume of 10 mM bis-tris-propane pH 7.2 and dialyzed against several changes of the same buffer. The dialyzed sample was then applied to a Q Sepharose (Pharmacia) column previously equilibrated with 10 mM bis-tris-propane

pH 7.2, washed with 75 mL of the same buffer, and eluted stepwise with 0.1 M and 1 M NaCl.

Table 3. Stepwise Elution of GVH Activity

Fraction	OD ₂₈₀	GVH Activity ($\mu\text{M F-}/\text{min}/50 \text{ uL}$)	Specific Activity ($\mu\text{M F-}/\text{min}/50 \text{ uL}/\text{OD}_{280}$)
10 mM BTP Wash	2.82	1.93	0.687
0.1 M NaCl Eluate	0.365	10.8	29.6
1 M NaCl Eluate	1.39	6.76	4.87

The 0.1 M NaCl eluate with the highest enzymatic activity was dialyzed overnight against 10 mM BTP pH 7.2 and again loaded on a second Q Sepharose column. After the nonbinding protein was removed by washing, elution was carried out with a linear gradient of 0 - 0.6 M NaCl in bis-tris propane buffer and analyzed for GVH activity. Figure 3 shows the GVH activity, DFP activity (OPAA) and OD₂₈₀ profile. The OPAA (DFP) activity peak is clearly distinct from the GVH activity peak, indicating these enzymes are not the same.

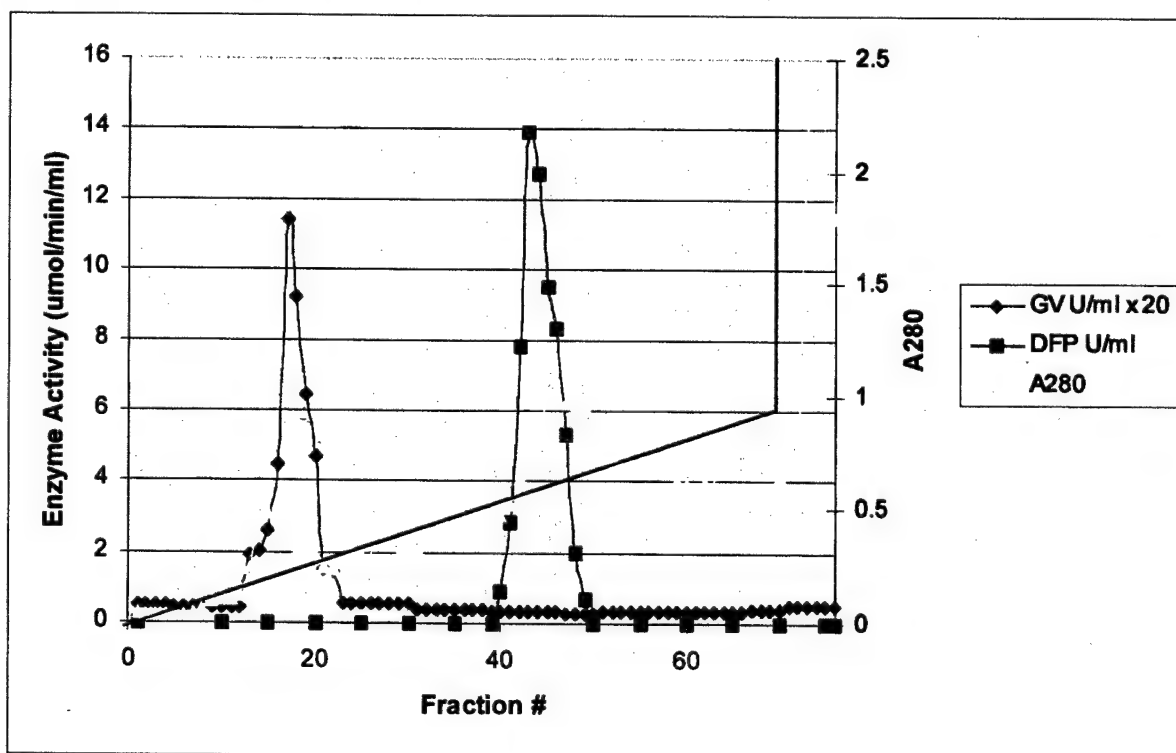


Figure 3. Purification of GVH on Q Sepharose Column with 0 - 1.0 M NaCl Gradient

The fractions with GVH activity (fraction number 16-20) were pooled and loaded on a second smaller (5 mL) Q Sepharose column and eluted with a 0-0.15 M NaCl gradient (Figure 4).

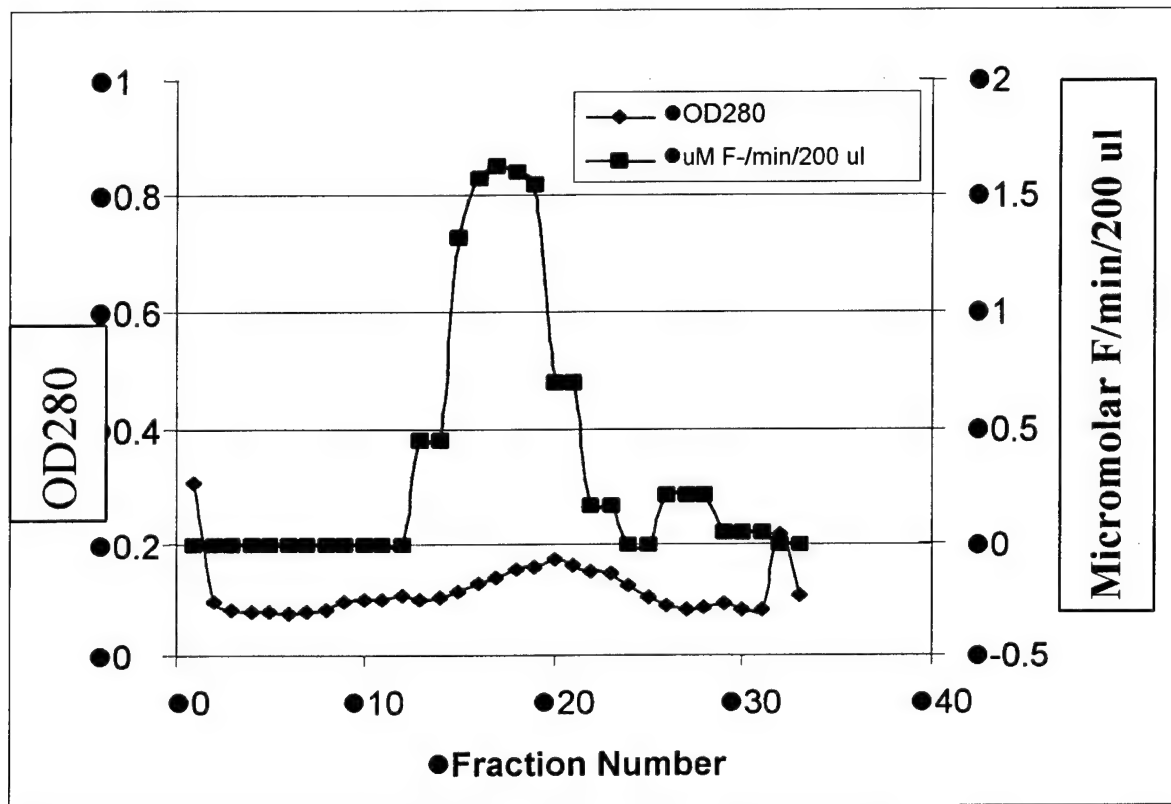


Figure 4. Purification of GVH on Second Q Sepharose Column

The enzyme fraction with highest activity (fraction #18) from this second Q Sepharose chromatogram was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Results (Figure 5) show a single band with a molecular weight of approximately 20 kilo Daltons (kDa).

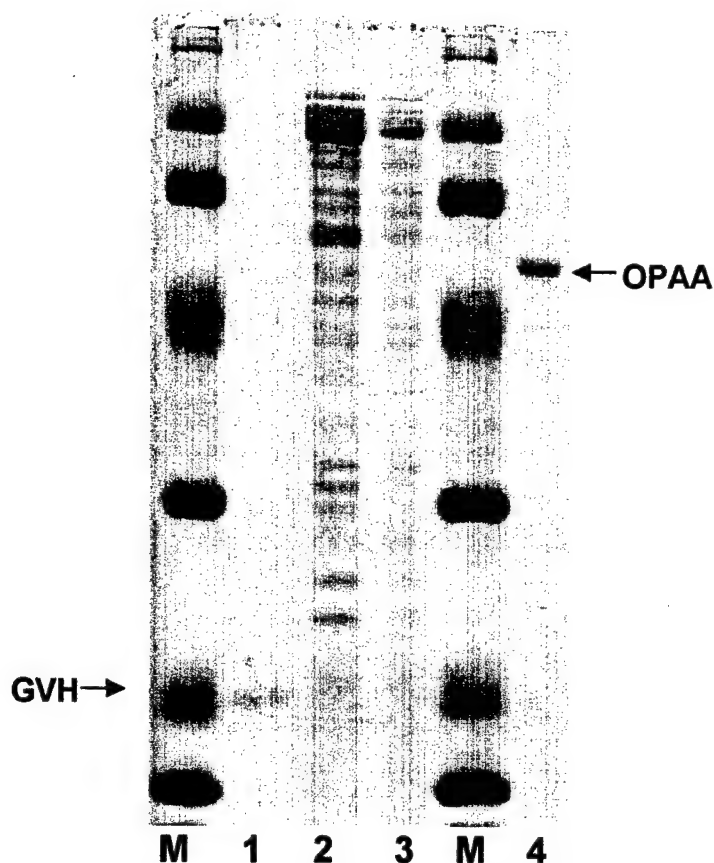


Figure 5. SDS-PAGE of Fractions from GV Degrading Enzyme Purification
Lanes 1 to 3 contain purified enzyme, 40-65% ammonium sulfate ppt,
and crude extract, respectively. M; molecular weight standards.

3.4. Enzyme Characterization.

3.4.1. pH Profile.

The most active fraction (partially purified enzyme) from the first Q Sepharose column was used to determine the optimal assays conditions for the enzyme. The profile of the enzyme activity versus pH (Figure 6) was obtained using 3 mM substrate at 25 °C in 50 mM bis-tris-propane buffer.

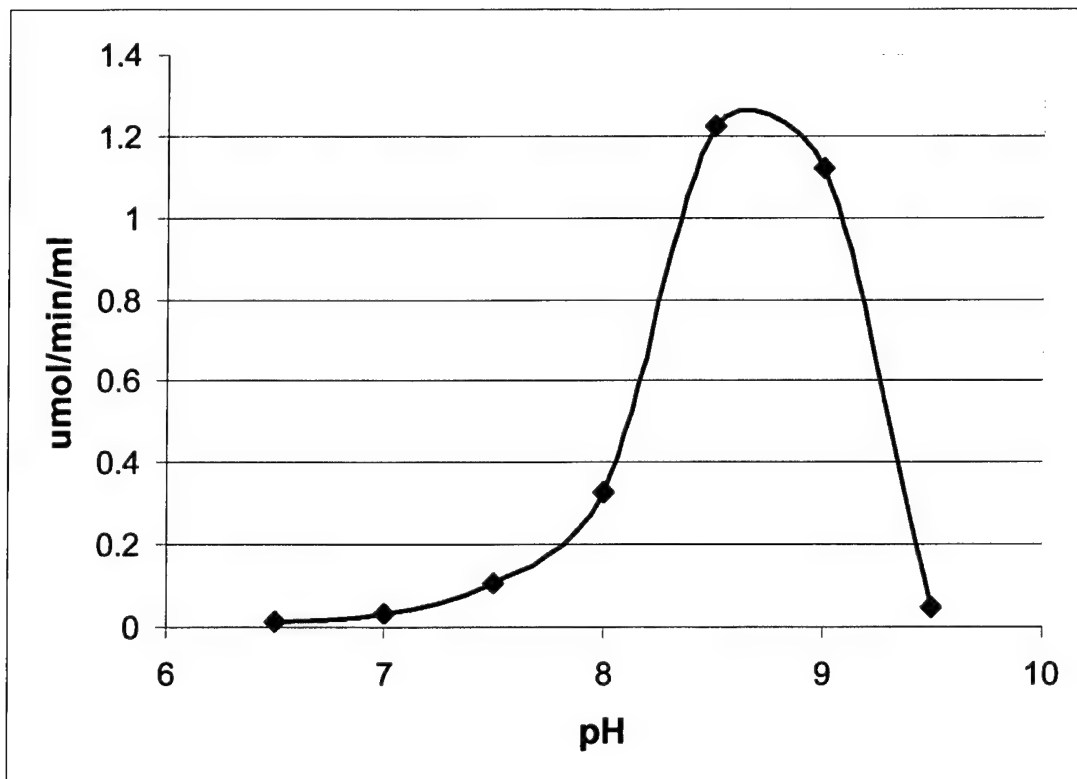


Figure 6. pH Profile of GVH

3.4.2. Temperature Profile.

Under the same condition used for the pH profile, a temperature profile was determined for the GVH enzyme at the optimal pH of 8.5 (Figure 7).

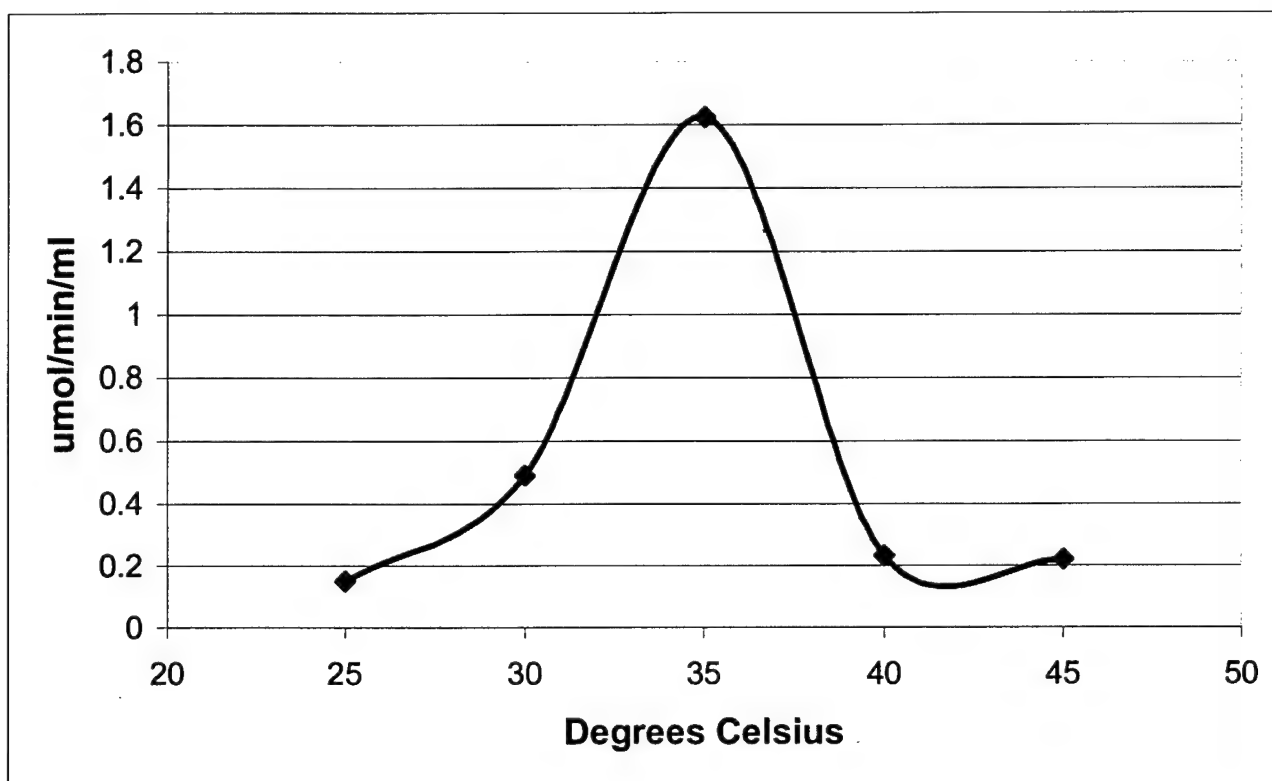


Figure 7. Temperature Profile of GVH

4. CONCLUSIONS

First report of enzymatic degradation of GV -demonstrated presence of GVH in several strains.

The *A. undina* GVH has been purified to apparent homogeneity (as determined by SDS-PAGE) following ammonium sulfate precipitation and two successive Q Sepharose chromatography steps.

The GVH enzyme has fairly narrow pH and temperature profiles with optima at approximately pH 8.5 and 35 °C, respectively.

Blank

LITERATURE CITED

1. Mulbry, W.W., Karns, J.S., Kearney, P.C., Nelson, J.O., McDaniel, C.S., and Wild, J.R., "Identification of a Plasmid-Borne Parathion Hydrolase Gene from *Flavobacterium* sp. by Southern Hybridization with *opd* from *Pseudomonas diminuta*," Appl. Environ. Microbiol. Vol. 51, pp 926-930 (1986).
2. McDaniel, C.S., Harper, L.L., and Wild, J.R., "Cloning and Sequencing of a Plasmid-Borne Gene (*opd*) Encoding a Phosphotriesterase," J. Bacteriol. Vol. 170, pp 2306-2311 (1988).
3. Dave, K.I., Miller, C.E., and Wild, J.R., "Characterization of Organophosphorus Hydrolases and the Genetic Manipulation of the Phosphotriesterase from *Pseudomonas diminuta*," Chem-Biol. Interactions Vol. 87, pp 55-68 (1993).
4. Rowland, S.S., Speedie, M.K., and Pogell, B.M., "Purification and Characterization of a Secreted Phosphotriesterase (Parathion Hydrolase) from *Streptomyces lividans*," Appl. Environ. Microbiol. Vol. 57, pp 440-444 (1991).
5. Dumas, D.P., Caldwell, S., Wild, J., and Raushel, F., "Purification and Properties of the Phosphotriesterase from *Pseudomonas diminuta*," J. Biol. Chem. Vol. 264, pp 19659-19665 (1989).
6. Dumas, D.P., Durst, H.D., Landis, W.G., Raushel, F.M., and Wild, J.R., "Inactivation of Organophosphorus Nerve Agents by the Phosphotriesterase from *Pseudomonas diminuta*," Arch. Biochem. Biophys. Vol. 277, pp 155-159 (1990).
7. DeFrank, J.J., Beaudry, W.T., Cheng, T.-c., Harvey, S.P., Stroup, A.N. and Szafraniec, L.L., "Screening of Halophilic Bacteria and *Alteromonas* Species for Organophosphorus Hydrolyzing Enzyme Activity," Chem. Biol. Interactions Vol. 87, pp 414-448 (1993).
8. DeFrank, J.J., Cheng, T.-c., Kolakowski, J.E. and Harvey, S.P., "Advances in the Biodegradation of Chemical Warfare Agents and Related Materials," Journal of Cellular Biochemistry Supplement Vol 0 (21A):41 (1995).
9. Serdar, C.M., Murdock, D.C., and Rohde, M., "Parathion Hydrolase Gene from *Pseudomonas diminuta* MG: Subcloning, Complete Nucleotide Sequence, and Expression of the Mature Portion of the Enzyme in *Escherichia coli*," Bio/Technology Vol. 7, pp 1151-1155 (1989).
10. Serdar, C.M., and Gibson, D.T., "Enzymatic Hydrolysis of Organophosphates: Cloning and Expression of a Parathion Hydrolase Gene from *Pseudomonas diminuta*," Bio/Technology Vol. 3, pp 567-571 (1985).

11. Cheng, T-c., Harvey, S.P., and Chen, G.L., "Cloning and Expression of a Gene Encoding a Bacterial Enzyme for Decontamination of Organophosphorus Nerve Agents and Nucleotide Sequence of the Enzyme.," Appl. Environ. Microbiol. Vol. 62, pp 1636-1641 (1996).
12. Ashani, Y., Shapira, S, Levy, D., and Wolfe, A.D., "Butyrylcholinesterase and Acetylcholinesterase Prophylaxis against Soman Poisoning in Mice," Biochem. Pharmacol. Vol. 41, pp 37-41 (1991).
13. Kolakowski, J.E., DeFrank, J.J., Harvey, S.P., Szafraniec, L.L., Beaudry, W.T., Lai, K., and Wild, J.R., "Enzymatic Hydrolysis of the Chemical Warfare Agent VX and its Neurotoxic Analogues by Organophosphorus Hydrolase," Biocatalysis and Biotransformation Vol. 15, pp 297-312 (1997).
14. Lai, K., Dave, K.I., Wild, J.R., Szafraniec, L.L., Beaudry, W.T., and Harvey, S.P., "Enzymatic Decontamination of Organophosphorus Chemical Agents by Genetic and Biochemical Manipulation of Organophosphorus Hydrolase," in Proceedings of the 1994 ERDEC Scientific Conference on Chemical and Biological Defense Research, 15-18 November 1994, ERDEC SP-036, U.S. Army Edgewood Research, Development and Engineering Center, Aberdeen Proving Ground, MD, May 1996, UNCLASSIFIED Report (AD A313 080).
15. ACTA MEDICA 39:27, 1996.
16. Petrikovics, I., Cheng, T-c, Papahadjopoulos, D., Hong, K., Yin, R., DeFrank, J. J., Jaing, J., Song, Z. H., McGuinn, W. D., Sylvester, D., Pei, L., Madec, J., Tamulinas, C., Jaszberenyi, J. C., Barcza, T., and Way, J. L., "Long Circulating Liposomes Encapsulating Organophosphorus Acid Anhydrolase in Diisopropylfluorophosphate," Antagonism Toxicological Sciences Vol. 57, pp 16-21 (2000).
17. Perrella, F.W., "EZ-FIT: A Practical Curve-Fitting Program for the Analysis of Enzyme Kinetic Data on IBM-PC Compatible Computers," Analytical Biochemistry Vol. 174, pp 437-447 (1988).

DEPARTMENT OF THE ARMY
CDR USASBCCOM
ATTN AMSSB SCI C
5183 BLACKHAWK ROAD
APG MD 21010-5424

OFFICIAL BUSINESS

STANDARD B